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Regulation of human erythropoiesis by activin A, BMP2, and BMP4, members of the TGFβ family

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Abstract

Activin A, BMP2, and BMP4, members of the TGFβ family, have been implicated in the regulation of hematopoiesis. Here we explore and compare, for the first time in human primary cells, the role of activin A, BMP2, and BMP4 during erythropoiesis. Using *in vitro* erythroid differentiation of $CD34^+$ primary cells, we obtained the main stages of early erythropoiesis, characterized at the molecular, biochemical, and functional levels. Our results indicate that BMP2 acts on early erythroid cells and activin A on a more differentiated population. We report an insight into the mechanism of commitment of erythropoiesis by activin A and BMP2 involving two key events, increase in EPO-R and decrease in GATA2 expression. Simultaneous addition of activin A with BMP molecules suggests that BMP2 and BMP4 differently affect activin A induction of erythropoiesis. Follistatin and FLRG proteins downmodulate the effects of activin A and BMP2 on erythroid maturation.

Keywords: Erythropoiesis; Activin; BMP; FLRG; Follistatin

Introduction

Hematopoiesis is sustained by uncommitted stem cells that give rise to progenitors capable of producing mature myeloid and lymphoid blood cells. This process is governed by a large number of hematopoietic-specific cytokines. These factors often act in cascade, directing cells toward either the cell cycle or differentiation. TGFβ is one of the key regulatory elements of the hematopoietic system [1–3]. The TGFβ family, which include TGFβ, activins, and bone morphogenetic proteins (BMPs), are secreted proteins that regulate numerous cellular responses, such as proliferation, differentiation, migration, and apoptosis. They also have critical roles during embryogenesis and in maintaining tissue homeostasis during adult life. Activin A is involved in the differentiation of hematopoietic progenitors and may be considered as a commitment factor [4–8]. In mouse embryonic stem cells, activin A has also been found to be involved in the development of hematopoietic cell formation [9]. The involvement of BMP molecules in the regulation of hematopoiesis on various levels of stem cell differentiation has recently emerged [10–14]. In particular, activin A [6,15,16] and BMP2 and BMP4, alone or in combination with activin A [9,12,17–20], have been involved in the regulation of erythropoiesis in various models. Active erythropoiesis occurs after erythroid commitment of undifferentiated pluripotent stem cells, which provide cells able to terminally differentiate into mature red blood cells. This process is controlled by the combined effects of growth factors (including the erythroid-specific cytokine erythropoietin) that permit cellular proliferation and lineage-specific gene regulation, such as GATA2, c-Myb, and EPO-R [21–23]. Erythroid differentiation is mainly characterized by the transient ability of cells to generate erythroid colonies in semisolid media and a progressive increase in specific erythroid marker expression (CD36, glycophorin A) [24].

The biological activity of activin A and BMP is antag-

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onized by binding with follistatin [25–27]. Recently, we and others have shown that FLRG (follistatin-related gene) acts as a binding protein for activin A and BMP2 and regulates their functions [28–30]. This suggests that FLRG, with follistatin, constitutes another partner in the extracellular control of the biological activity of activin A and BMP molecules. In the hematopoietic system, we have observed that FLRG and activin A are expressed in the same cells and that their expression is regulated by TGFβ [29,31]. It is therefore likely that secreted FLRG, in association with activin A, plays an important role in hematopoiesis.

In this study, we explored the role and the mechanism of action of activin A, BMP2, and BMP4, and of their regulators, during human erythropoiesis. We first characterized the different stages of the erythroid differentiation obtained *in vitro* at the molecular, biochemical, and functional levels. The addition of activin A and BMP2 in this system indicates that these molecules are involved in the regulation of human erythropoiesis, follistatin and FLRG modulating their effects.

Materials and methods

Proteins

Recombinant human activin, BMP2, BMP4, and follistatin were commercially available (R&D Systems). Human recombinant FLRG protein was produced in Sf9 cells using a baculovirus expression system [28]. The secreted recombinant protein was purified by affinity chromatography using an anti-FLRG-purified antibody linked to NHS-activated Sepharose (Amersham Pharmacia Biotech). The purity of the protein was analyzed on SDS–PAGE gels by silver staining and by immunoblot analysis with an anti-FLRG antibody (data not shown). The concentration for each cytokine was chosen regarding previous studies performed on various system, in particular in erythropoiesis [6, 10,12,28,32].

Primary cells and culture conditions

Peripheral blood and normal bone marrow samples were obtained from consenting donors. Mononuclear cells, separated by a Ficoll gradient (Bio-Whittaker), were subjected to CD34 immunomagnetic bead separation (Dynal). The purity of the isolated $CD34^+$ population, checked by flow cytometry, was over 95%. Early erythropoiesis was obtained as previously described [24]. Briefly, $CD34^+$ cells were cultured at a density of $2\ 10^5$ cells/ml in serum-free Iscove's medium (Gibco/BRL) in the presence of 15% BIT (5% BSA, 50 μg/ml of bovine pancreatic insulin, 2 mM L-glutamine) supplemented with 20 ng/ml of IL6, 25 ng/ml of stem cell factor, and 10 ng/ml of IL3 (R&D Systems). After 7 days of culture, $CD36^+$ cells were selected using indirect immunomagnetic bead separation (Dynal) with a monoclonal anti-CD36 antibody (Clone FA6-152, Coulter). Cells were then further cultured for another 4 to 20 days in serum-free Iscove's medium (Gibco/BRL) in the presence of 15% BIT supplemented with 20 ng/ml of IL6, 25 ng/ml of stem cell factor, 10 ng/ml of IL3 (R&D Systems), and 3 U/ml of human erythropoietin (EPO).

Immunophenotyping assay

The cells were stained using 0.1 μg of antibody per 10^6 cells and incubated for 30 min at 4°C. After a final wash, flow cytometry analysis was performed using a Facscalibur cell analyzer (Becton–Dickinson). The stages of erythropoiesis obtained were monitored by phenotypic analysis using CD36-FITC (Immunotech) and glycophorin-A-PE (Caltag) as reported [24]. The antibodies used for phenotyping were of the IgG_1 subclass.

Cell morphology and nuclear size measurement

To analyze cell morphology, cells were cytocentrifuged (Shandon) onto slides and stained with May–Grunwald Giemsa. Cytological analysis was realized on cells harvested at various times of culture to estimate the corresponding stage of differentiation. Nuclear sizes of a thousand cells per condition were measured by the image analysis system Quantimet 600 (Leica) using the cytospin slides previously used for morphological analysis.

Progenitor assays

The number of colony-forming cells (CFC) was determined by suspending them in Iscove's methylcellulose medium containing erythropoietin, stem cell factor, IL6, GM-CSF, G-CSF, and IL3 (Stem Cell Technologies) and plating them in 35-mm petri dishes. After 16 days of incubation at 37°C, the colonies were counted *in situ* using standard scoring criteria to identify erythroid, granulocyte–macrophage, and mixed colonies (BFU-E, CFU-E, CFU-GM, and CFU-GEMM, respectively). Distinctions between CFU-E, early, and late BFU-E were based on the size and numbers of erythroid clusters. The most immature erythroid (called early BFU-E) progenitors generated large colonies with a high number of clusters per colony (9 to 16 and more), followed by the named late BFU-E (3 to 8 erythroid clusters per colony). Finally the most differentiated erythroid progenitor (CFU-E) is made up of a very small colony, including 1 to 2 clusters per colony.

RNA isolation and analysis

Total cellular RNA was isolated from samples lysed in TRI REAGENT (Sigma). For RT–PCR analysis, cDNA was synthesized using Superscript II RNase H reverse transcriptase (Gibco/BRL), in accordance with the manufacturer's instructions, in a final volume of 15 μl. PCR was performed using 1 μl of cDNA made from 1 μg of RNA. The reaction was carried out in a final volume of 50 μl (20 mM Tris–HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM each dNTP (Roche); 2.5 Units of Platinium *Taq* polymerase (Gibco/BRL), and specific gene primers (Table 1) were simultaneously added with GAPDH primers, used as an

Table 1
Oligonucleotide primers used for gene expression analysis by RT–PCR

Gene	Sense primer	Antisense primer	Size (bp)
FLRG	ACG TCA CCT ACA TCT CCT CG	CAC GAA GTT CTC TTC CTC TTC	145
Follistatin	CCA TGG GGA ACT GCT GGC TCC GTC AA	CCA GGT CCA CAG TCC ACG TT	220
β-Globin	GTC CTT TGG GGA TCT GTC CA	GTG GGG TGA ATT CTT TGC CA	239
E-PBGD	TGC AGC GGC AGC AAC AGC AG	CTG GCT CTT GCG GGT ACC CA	150
GATA 2	CCC TAA GCA GCG CAG CAA GAC	TGA CTT CTC CTG CAT GCA CT	439
EPO-R	TGA GAC ACC CAT GAC GTC TCA	TGT CCA GCA CCA GAT AGG TA	608
Act RIIA	ATG CTC TGT GAA ACC ATT GAA G	TAG ACT AGA TTC TTT GGG AGG A	180
Act RIIB	GAA CAT GAC GGC GCC CTG GG	TGA TCT CCA GCA GCT GCA GT	590
Act RIA	CTG GCC AAG CTG TGG AGT GCT GCC AA	GTA CTG GAG TGT CTA GAG GTC ATG	570
Act RIB	TAT GGC GGA GTC GGC CGG A	GAT CGT AGA CGA GAT CCT GGA GCG	545
BMP RII	GCC ATG ATG AAG GTG TTC TG	AGT GCC TCC TTC TGC AAG GT	440
BMP RIA	GCT CAT CGA GAC CTA AAG AG	GTC TGG AGG CTG GAT TGT GG	460
BMP RIB	AGA AGT TAC GCC CCT CAT TC	TTG ATG TCT TTT GCT CTG CC	250
c-Myb	ACA GCA TAT ATA GCA GTG ACG	AGC CTG AGC AAA ACC CAT CAA	659
$P45^{NFE2}$	GAG ATG GAA CTG ACT TGG CAG	GGA CAT GGG ATG TGG ATG CTG	210
$p27^{Kip1}$	AAA CGT GCG AGT GTC TAA CGG GA	CGC TTC CTT ATT CCT GCG CAT TG	454
Bcl-XL	TGC GCA CAG CAG CAG TTT GG	CTC GGC TGC TGC ATT GTT CC	400
GAPDH	CGG AGT CAA CGG ATT TGG TCG TAT	AGC CTT CTC CAT GGT GGT GAA GAC	300

Act R, activin receptor; BMP R, BMP receptor; EPO-R, erythropoietin receptor; E-PBGD, erythroid-specific isoform of porphobilinogen deaminase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

internal control. The relative proportions of primers used for the gene of interest and GAPDH were optimized for each combination so as to obtain the maximum amplification of the gene of interest in comparison to GAPDH. Cell- and reverse transcriptase-free samples were used as negative controls, and the specificity of each product was verified by Southern blot analysis with an internal oligoprobe. To compare the relative levels of expression of the genes among the various samples, the volume of the PCR product loaded onto 2 or 3% agarose gels was adjusted in such a way as to produce an equal GAPDH signal.

Western blot analysis

Cell lysates were prepared using solubilization buffer (50 mM Hepes buffer, pH 7.4, 100 mM NaF, 10 mM NaPPi, 2 mM Na_3VO_4, 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml of leupeptin, and 10 μg/ml of aprotinin) containing 0.5% NP-40. Protein concentrations were determinate using a BCA protein assay (Pierce). Cell lysates (5 μg of protein per lane) were separated by 12% SDS–PAGE and transferred onto membranes (Immobilon-P, Millipore). The filters were incubated with rabbit anti-hemoglobin or anti-Bcl-XL (Dako) or with the monoclonal mouse anti-actin (Roche) or p27Kip1 (Santa Cruz) antibodies, then incubated with, respectively, a swine anti-rabbit or a rabbit anti-mouse peroxidase-conjugated secondary antibody (Dako), and revealed with an ECL detection kit (Amersham Pharmacia Biotech).

Statistics

Differences between progenitors numbers in different treatment groups were assessed using the Student *t* test. As the cell nuclear size values did not follow a normal distribution we used the nonparametric Mann–Whithney statistical test to compare treated cells to our control.

Results

In vitro model of early human erythropoiesis from $CD34^+$ progenitors

In order to study human erythropoiesis, we used primary progenitors to generate an *in vitro* model representing the main stages of differentiation. Purified $CD34^+$ cells were cultured in serum-free media in the presence of IL6, stem cell factor (SCF), and IL3. After 7 days of culture, $CD36^+$ cells, representing an enriched erythroid population [24], were selected and further cultured for another 20-day period in the same media supplemented with IL6, SCF, IL3, and EPO. In order to monitor the evolution of the *in vitro* erythropoiesis, we assayed colony formation, phenotype, and cell nuclear size. We measured cell function at various stages of the culture by evaluating clonogenic ability using the CFC assay. As indicated by the total CFC number (Fig. 1A), the time frame for detecting clonogenic cells is limited to early time points (up to 7 days before the $CD36^+$ selection). As expected, after the $CD36^+$ selection, we observed a rapid decrease in the CFU-GM and CFU-GEMM colony numbers with cell maturation (Fig. 1A). As shown in the right panel of Fig. 1A, the different types of erythroid colonies appeared sequentially. The most immature early BFU-E is the first detected, with a peak on day 4; then they rapidly disappear, followed by the late BFU-E, which also peak on day 4, presenting, however, a more progressive decrease. And finally the most differentiated erythroid progenitors (CFU-E) start to appear on day 4 but peak late on

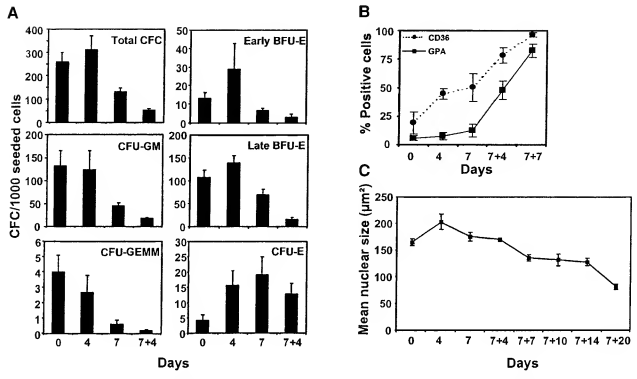

Fig. 1. Functional, phenotypic, and morphologic variation during *in vitro* erythropoiesis. $CD34^+$ cells were cultured as described under Materials and Methods. At the indicated time throughout the culture, cells were harvested and analyzed for colony-forming ability, phenotype, and morphology. (A) Clonogenic ability of cells during *in vitro* erythropoiesis. Cells were harvested and analyzed for their clonogenic content using the CFC assay. The colonies were counted using standard scoring criteria to identify erythroid (BFU-E, CFU-E), granulocyte–macrophage (CFU-GM), and mixed granulocyte–erythroid monocyte–megakaryocyte colonies (CFU-GEMM). The results are expressed as CFC/1000 seeded cells and represent the mean value ± SEM of six experiments carried out with different bone marrow or blood samples. (B) Phenotypic analysis of erythroid-specific markers, CD36 and glycophorin A (GPA), is presented as the percentage of positive cells ± SEM of five independent experiments. (C) Cell morphology was analyzed on cytocentrifuged cells stained with histological dyes. On the same slides, the nuclear size on 1000 cells per time point was measured using an image analysis system. Results are presented as the mean value ± SEM of nuclear size and are representative of three independent experiments.

day 7 and then slowly decrease. Phenotyping analysis using erythroid-specific markers indicates that expression of the CD36 molecule is induced by cell differentiation, while that of glycophorin A (GPA) appears later during erythropoiesis (Fig. 1B), thus confirming that these molecules represent, respectively, early and late erythroid markers. Cytological analysis was realized on cells harvested at various times of culture to estimate the corresponding stage of differentiation. We observed a progressive enrichment from the proerythroblast stage on day 7+4 up to the erythroblast acidophil stage after 7+20 days of culture. Measurement of nuclear size has been described to characterize one differentiation event observed during erythroid maturation [33]. The nuclear size of cells, measured on the cytospin slides previously morphologically analyzed, progressively decreases with maturation after a brief increase, due likely to the activation of the cells by culture condition (Fig. 1C).

Altogether, the sequential detection of erythroid colonies, the increase in erythroid markers, and the decrease in cell nuclear size indicate that this *in vitro* erythropoiesis system accurately represents the major steps of early erythroid maturation.

Gene expression during in vitro erythropoiesis

To further characterize each step of erythroid maturation, we evaluated by RT–PCR the expression of various genes involved in the regulation of erythropoiesis. For that purpose, cells were harvested at different culture times and RNA was extracted and used for RT–PCR study. The first group of genes studied includes genes specifically involved in erythropoiesis, such as the erythropoietin receptor (EPO-R), the erythroid-specific isoform of the third enzyme of the heme biosynthetic pathway, namely, porphobilinogen deaminase (E-PBGD), and the β-globin chain, a constitutive element of hemoglobin. We observed a regular increase in EPO-R expression that peaks between 7+4 and 7+10 days of culture, respectively, corresponding to the CFU-E and basophil erythroblast stages (Fig. 2A) The expression of EPO-R then slowly decreases. A regular increase in the expression of E-PBGD and β-globin during maturation (Fig. 2A) correlates with an accumulation of hemoglobin in the cells [34]. We monitored the expression of several transcription factors that played a role during erythropoiesis. The early transcription factors GATA2 and c-Myb are highly expressed during the first part of the culture and then progressively decrease (Fig. 2A). We first report a rapid in-

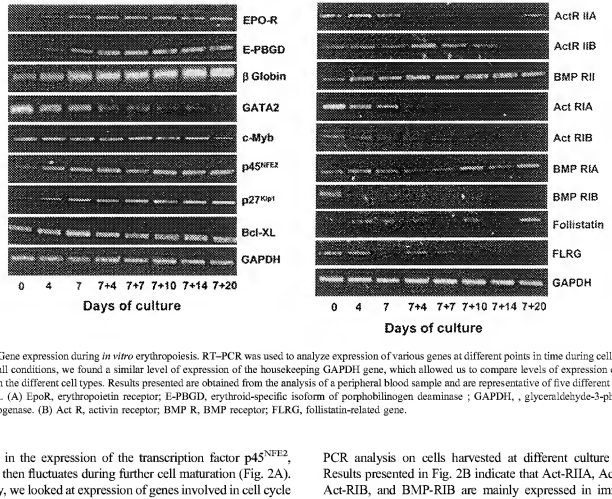

Fig. 2. Gene expression during *in vitro* erythropoiesis. RT–PCR was used to analyze expression of various genes at different points in time during cell culture. Under all conditions, we found a similar level of expression of the housekeeping GAPDH gene, which allowed us to compare levels of expression of genes between the different cell types. Results presented are obtained from the analysis of a peripheral blood sample and are representative of five different samples assayed. (A) EpoR, erythropoietin receptor; E-PBGD, erythroid-specific isoform of porphobilinogen deaminase ; GAPDH, , glyceraldehyde-3-phosphate dehydrogenase. (B) Act R, activin receptor; BMP R, BMP receptor; FLRG, follistatin-related gene.

crease in the expression of the transcription factor $p45^{NFE2}$, which then fluctuates during further cell maturation (Fig. 2A). Finally, we looked at expression of genes involved in cell cycle and apoptosis mechanisms and implicated during erythropoiesis: $p27^{Kip1}$, an inhibitor of cyclin-dependent kinase activity, and Bcl-XL, a member of the Bcl2 family protein. We observed a rapid increase in $p27^{Kip1}$ with cell maturation, while expression of Bcl-XL, after a rapid increase, fluctuated during cell maturation (Fig. 2A). Altogether these results correlate with a specific erythroid maturation previously observed in various cellular models [21–23,35–38].

In order to explore the roles and the mechanisms of action of activin A, BMP2, and BMP4 during human erythropoiesis, we first characterized the expression patterns of their receptors and of their regulators, follistatin and FLRG. As members of the TGFβ family, both BMP and activin proteins transduce their signals by inducing the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors [39]. Activin supposedly signals only via combinations of activin-specific receptors, mainly Act-RIIA, Act-RIIB, and Act-RIA (Alk2). On the other hand, BMP molecules are able to signal not only through BMP-RII, BMP-RIA (Alk3), and BMP-RIB (Alk6) but also through Act-RIIA, Act-RIIB, and Act-RIA [40]. In order to determine whether erythroid primary cells are able to respond to activin and BMP, we analyzed the expression of their various receptors during erythropoiesis. The expression of these genes was realized by RT–PCR analysis on cells harvested at different culture times. Results presented in Fig. 2B indicate that Act-RIIA, Act-RIA, Act-RIB, and BMP-RIB are mainly expressed in immature cells, while Act-RIIB, BMP-RII, and BMP-RIA are expressed throughout erythropoiesis but with a variable expression during cell differentiation. As regards the expression of the regulators, it appears that their expression is almost inversely related as we observed the highest expression of FLRG in the most immature and of follistatin in the most mature erythroid cells.

In summary, we show that *in vitro* human erythropoiesis can be characterized by an increase in EPO-R, E-PBGD, β-globin, $p45^{NFE2}$, $p27^{Kip1}$, and Bcl-XL gene expression and a decrease in GATA2 and c-Myb gene expression. Our results also indicate that erythroid cells at various stages of their maturation possessed different receptors for activin and BMP and this therefore gave them the ability to respond to activin and BMP proteins. Here, we report for the first time the simultaneous analysis of morphologic, molecular, and functional parameters throughout the erythropoiesis of primary human cells.

Effects of activin A, BMP2, and BMP4 on human erythropoiesis

In order to explore the role of activin A, BMP2, and BMP4 during early human erythropoiesis, $CD34^+$ cells were cultured for 4 days in serum-free medium in the

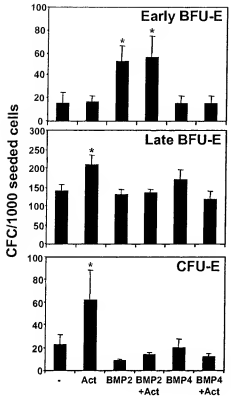

Fig. 3. Effects of activin, BMP2, and BMP4 on clonogenic erythroid precursors. $CD34^+$ cells, obtained from different bone marrow or blood samples, were incubated at 2×10^5 per milliliter in serum-free IMDM, 15% BIT supplemented with IL6, stem cell factor, and IL3 in the presence or the absence (−) of 40 ng/ml of activin A (Act), BMP2, or BMP4 alone or in combination. After 4 days, cells were assessed for the proportion of clonogenic cells by a CFC assay. The results, which are expressed as CFC/1000 seeded cells, represent the mean value ± SEM of nine, five, or seven experiments for activin, BMP2, or BMP4, respectively. Differences that are statistically significant ($P < 0.05$) in comparison with nontreated cells are indicated by an asterisk.

presence or the absence of activin A (40 ng/ml), BMP2 (40 ng/ml), or BMP4 (40 ng/ml) alone or in combination. Cells were then harvested and various parameters were analyzed as described above. No significant effects were observed on expression of CD36 and GPA markers in the presence of activin A, BMP2, and BMP4 molecules either alone or in combination (data not shown). This is not surprising, given the slow expression modulation of these markers between days 4 and 7 of *in vitro* culture and the absence of EPO in the culture media (Fig. 1B). We assayed the biological function of treated erythroid cells by measuring their clonogenic potential using the CFC assay. No significant effect was seen on CFU-GM and CFU-GEMM colonies (data not shown). Activin A increases the detection of both late BFU-E and CFU-E colonies (Fig. 3). Inversely, BMP2 increases the most immature erythroid colonies, the early BFU-E, and has little or no effect on late BFU-E and CFU-E colonies. These results suggest that BMP2 acts on a more immature erythroid population than activin A. BMP4 alone has no significant effect on either type of erythroid colony. In order to verify that results obtained with the two different sample origins (blood or marrow) are equivalent, we analyzed separately the data set from each source of sample. Results presented in Table 2 confirmed that similar effects are observed by activin A, BMP2, or BMP4 treatment of $CD34^+$ purified cells from either mobilized peripheral blood or normal bone marrow. This indicate that these two cell populations behave the same way under the influence of the tested agents. We therefore combined the data obtained from these two type of cells in the following experiments.

We also analyzed the cell nuclear size as a marker of advanced erythroid cell differentiation. We then observed a strong decrease in the cell nuclear size of activin-A-treated cells, while no significant effect was obtained in the presence of BMP2 or BMP4 alone (Table 3). Together with the effect on erythroid colonies, our data suggest that activin A is a more potent inducer of human erythroid differentiation than BMP2 or BMP4 proteins. Interestingly, in the presence of BMP4 the activin A effect on late BFU-E and CFU-E colonies is no longer observed (Fig. 3), while the cell nuclear size decrease is still observed and even amplified (Table 3). These results might indicate that BMP4 cooper-

Table 2
Effect of activin, BMP2, and BMP4 on erythroid colonies obtained from mobilized peripheral or normal bone marrow $CD34^+$ cells

Treatment	None	Activin A	BMP2	BMP4
Peripheral blood				
Early BFU-E	23 ± 13	18 ± 8	65 ± 38	13 ± 8
Late BFU-E	162 ± 14	224 ± 37	131 ± 19	170 ± 31
CFU-E	28 ± 12	78 ± 45	10 ± 2	25 ± 10
(*n*)	(6)	(6)	(3)	(5)
Bone marrow				
Early BFU-E	12 ± 2	14 ± 3	61 ± 21	19 ± 7
Late BFU-E	101 ± 12	208 ± 19	112 ± 11	136 ± 21
CFU-E	25 ± 6	66 ± 15	19 ± 6	20 ± 7
(*n*)	(6)	(6)	(5)	(5)

$CD34^+$ cells isolated from either peripheral blood or normal bone marrow were incubated in the presence or the absence of 40 ng/ml of activin A, BMP2, or BMP4. After 4 days, cells were assessed for the proportion of erythroid colonies by a CFC assay. The results are expressed as CFC/1000 seeded cells ± SEM of the indicated number of experiments (*n*).

Table 3
Effect of activin, BMP2, and BMP4 on cell nuclear size

Treatment	Nuclear size ($\mu m^2 \pm$ SE)
None	277 ± 3.5
Activin	254 ± 2.8*
Activin + follistatin	273 ± 3.2
Activin + FLRG	272 ± 3.1
BMP2	276 ± 3.2
BMP2 + activin	292 ± 3.5*
BMP4	264 ± 3.2
BMP4 + activin	236 ± 3.8*

$CD34^+$ cells were incubated in the presence or the absence of activin (40 ng/ml) alone or combined with follistatin (100 ng/ml) or FLRG (100 ng/ml). Cells were also treated with BMP2 (40 ng/ml) or BMP4 (40 ng/ml) alone or in combination with activin A. After 4 days, cells were assessed for nuclear size by image analysis as described under Materials and Methods. Data are expressed as means of the nuclear size in $\mu m^2 \pm$ standard error (SE), measured on more than 1000 cells. Results shown are representative of three independent experiments, each performed with a different $CD34^+$ sample. We assayed significant differences ($P < 0.05$) from nontreated cells using the nonparametric Mann–Whitney statistical test, indicated by asterisks.

but decreases GATA2 expression (Fig. 4A) and increases Hb and Bcl-XL, although to a lesser extent than that with activin A (Fig. 4B). As regards the simultaneous addition of activin and BMP2, the expression of EPO-R, Hb, and $p27^{Kip1}$ is the same as with activin alone. BMP2 accentuates the effects of activin on Bcl-XL expression, while it attenuates the decrease of GATA2 expression. On the other hand, BMP4 reduces the expression of EPO-R and $p27^{Kip1}$ expression, accentuates the Bcl-XL expression, and has no effect on GATA2 and Hb expression induced by activin A.

In summary, our molecular, morphologic, and functional analyses show that activin A and BMP2 alone significantly regulate human erythropoiesis by inducing various and independent effects on erythroid precursors. It also suggests that when added simultaneously, BMP2 seems to overcome activin A's biological effects (CFC, cell nuclear size) without altering most of the gene expression modulated by activin A. On the other hand, BMP4 seems to accentuate the activin-A-mediated effects on erythropoiesis.

ates with activin to accelerate erythropoiesis up to a stage where the cells ability to from colonies has diminished and the cell nuclear size continues to decrease. On the other hand, in the presence of both activin A and BMP2, the effect on erythroid colonies appears to be the same as that with BMP2 alone (Fig. 3). Moreover, the cell nuclear size of cells treated with both molecules does not decrease as with activin alone, but increases compared to nontreated cells. These observations might indicate that BMP2 is able to compete and overcome activin's biological effects.

We observed that activin A and BMP2 alone significantly regulate human erythropoiesis. Our data also suggest different effects of the BMP molecules on activin-A-mediated biological activities.

Effects of activin A, BMP2, and BMP4 on expression of genes involved in erythropoiesis

To characterize and confirm the effect of activin A, BMP2, and BMP4 on human erythropoiesis, we conducted semi-quantitative RT–PCR comparisons of gene expression previously monitored during *in vitro* differentiation. Activin A induced a significant increase in EPO-R expression and inversely it decreased GATA2 expression (Fig. 4A). No other significant modulations of gene expression were observed (data not shown). Western blot analysis showed that activin A increased the expression of hemoglobin (Hb), $p27^{Kip1}$, and Bcl-XL proteins (Fig. 4B). BMP2 had similar effects to activin A, though less pronounced, on GATA2, EPO-R, and Hb expression, while it had no effect on $p27^{Kip1}$ and Bcl-XL (Fig. 4). Again, these results indicate that activin A is a stronger regulator of human erythropoiesis than BMP2. BMP4 has no effect on EPO-R expression

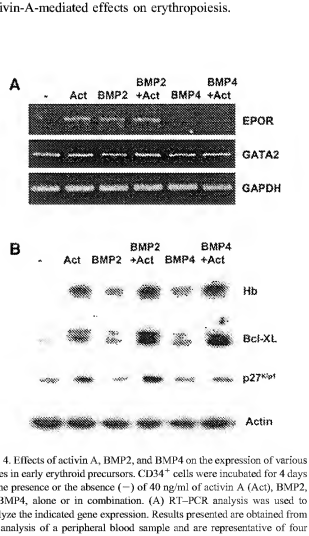

Fig. 4. Effects of activin A, BMP2, and BMP4 on the expression of various genes in early erythroid precursors. $CD34^+$ cells were incubated for 4 days in the presence or the absence (−) of 40 ng/ml of activin A (Act), BMP2, or BMP4, alone or in combination. (A) RT–PCR analysis was used to analyze the indicated gene expression. Results presented are obtained from the analysis of a peripheral blood sample and are representative of four different samples assayed. (B) Total cellular proteins (5 μg) were analyzed by Western blotting using the indicated antibody. The same blot was probed for actin to control for equal protein loading. Results presented were obtained from the analysis of a peripheral blood sample and are representative of two different samples assayed.

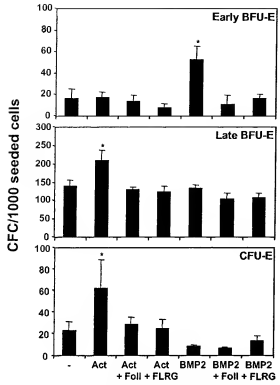

Fig. 5. Effects of activin A and BMP2 on clonogenic erythroid precursors are modulated by follistatin and FLRG. $CD34^+$ cells were incubated in the presence or the absence (−) of 40 ng/ml of activin A (Act) or BMP2 alone or in combination with 100 ng/ml of follistatin (Foll) or FLRG. After 4 days, cells were assessed for the proportion of erythroid colonies by a CFC assay. The results, which are expressed as CFC/1000 seeded cells, represent the mean value ± SEM of six experiments carried out with different bone marrow or blood samples. Differences that are statistically significant ($P < 0.05$) relative to nontreated cells are indicated by an asterisk.

Follistatin and FLRG regulate the effects of activin A and BMP2 on human erythropoiesis

Finally, we assayed whether follistatin and FLRG modulate the effects of activin A and BMP2 on human erythropoiesis. We choose these two modulators within the multiple regulators of the BMP as they are also involved in the regulation of activin's biological effects. $CD34^+$ cells were incubated 4 days in the presence or the absence of activin A (40 ng/ml) and BMP2 (40 ng/ml) alone or in combination with follistatin (100 ng/ml) or FLRG (100 ng/ml). Results presented in Table 3 show that either follistatin or FLRG proteins cancel the decrease in cell nuclear size induced by activin A. These molecules also block the biological activity of activin A, as indicated by the level of both late BFU-E and CFU-E, comparable to that in nontreated cells (Fig. 5). Similarly, the increase in early BFU-E induced by BMP2 is abolished by the addition of follistatin or FLRG (Fig. 5). We then evaluated whether follistatin and FLRG modulate the effects of activin A and BMP2 on the gene expression involved in erythropoiesis. Follistatin and FLRG block BMP2- and Activin-A-induced expression of EPO-R (Fig. 6A) and Hb (Fig. 6B). Interestingly, the inhibition by FLRG of EPO-R expression is stronger than that by follistatin. On the other hand, only follistatin inhibits activin-, and to a lesser extent BMP2-, induced GATA-2 decreased expression (Fig. 6A).

These results confirm that both follistatin and FLRG proteins can modulate the biological activity of activin A and BMP2. Moreover, they indicate that the effects of these two antagonists on activin-A- or BMP2-modulated gene expression are different from one target to another. This suggests that these regulators are not redundant and might act on different signaling pathways.

Discussion

We investigated here the role and mechanism of action of activin A, BMP2, and BMP4 during human erythroid cell maturation. For that purpose, we used an *in vitro* differentiation system initiated with primary purified human $CD34^+$ cells cultivated in a serum-free medium. This system generated enough primary cells to allow us to perform various molecular, biochemical, morphologic, and functional assays on the same cells at a precise step of erythroid differentiation. It enabled us to accurately reproduce *in vitro* the major steps of early erythroid maturation. Indeed, this is indicated by the sequential detection of the various classes of erythroid colonies (early and late BFU-E, CFU-E), the modulation of specific cell surface

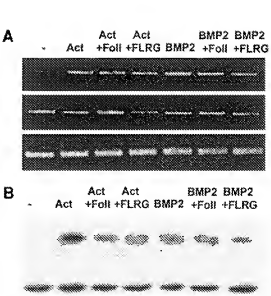

Fig. 6. Modulation by follistatin and FLRG of activin A and BMP2 effects on gene expression. $CD34^+$ cells were incubated for 4 days in the presence or the absence (−) of 40 ng/ml of activin A (Act) or BMP2 alone or in combination with 100 ng/ml of follistatin (Foll) or FLRG. (A) RT–PCR analysis was used to analyze the indicated gene expression. Results presented are obtained from the analysis of a peripheral blood sample and are representative of three different samples assayed. (B) Total cellular proteins (5 μg) were analyzed by Western blotting using the hemoglobin antibody. The same blot was probed for actin to control for equal protein loading. Results presented were obtained from the analysis of a peripheral blood sample and are representative of two different samples assayed.

membrane markers (CD36, GPA), the decrease in cell nuclear size, the increase in EPO-R, E-PBGD, β-globin, $p45^{NFE2}$, $p27^{Kip1}$, and Bcl-XL expression, and a decrease in GATA2 and c-Myb expression. We are now able to draw a pattern of various parameters, including specific gene expression, for a given human erythroid subpopulation.

As members of the TGFβ family, both BMP and activin proteins transduce their signals by inducing the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors [39]. In order to determine whether erythroid primary cells are able to respond to activin and BMP, we analyzed the expression of their different receptors during erythropoiesis. We observed that erythroid cells at various stages of their maturation possess different receptors for activin and BMP and therefore can potentially respond to such molecules. As most activin and BMP receptors are present in immature cells, we evaluated the effect of these cytokines at one of the earliest stage of erythropoiesis. Activin A decreased cell nuclear size and GATA2 gene expression. It also increased the expression of EPO-R, Hb, Bcl-XL, and $p27^{Kip1}$ proteins. On a biological level, activin A increased the detection of both the late BFU-E and the CFU-E colonies. BMP2 has effects that are similar to but less pronounced than those of activin A on EPO-R, GATA2, and Hb expression. While activin A increased late BFU-E and CFU-E, BMP2 increased the most immature early BFU-E colonies and had no significant effect on other erythroid colonies. These results demonstrate that activin A and BMP2 alone significantly regulate human erythropoiesis by inducing various molecular and functional effects. They also suggest that BMP2 acts on a more immature erythroid population than activin A. And finally, activin A appeared to be a stronger regulator of human erythropoiesis than BMP2.

In this study we demonstrate that the addition of activin A or BMP2 to primary human precursors induced their commitment toward the erythroid lineage, as suggested by previous data obtained on erythroid murine cell lines with activin A [8]. We report here for the first time the modulation by activin A and BMP2 of several genes that sign the engagement of the cells in erythroid differentiation. Indeed, we show that activin A or BMP2 treatment, in the absence of EPO, characteristically induced EPO-R expression and decreased GATA2 expression. These events are correlated with an accelerated detection of late BFU-E and CFU-E colonies after activin A treatment and with an accelerated detection of early BFU-E with BMP2. Our results give an insight into the activin A and BMP2 mechanism of action for cell commitment toward erythroid cells. Moreover, these data argue in favor of a sensitization of cells to the survival factor EPO further needed to progress in cell maturation [41]. Inversely to TGFβ [42], we cannot exclude that erythroid cells themselves produced EPO and that they stimulated their own erythroid differentiation in an autocrine manner [43]. Our results show that activin A and BMP2 do not simply induce EPO-R expression, but also decrease GATA2 expression, which constitutes a required condition for erythroid differentiation [44]. The other effects, such as induction of Hb, $p27^{Kip1}$, and Bcl-XL, can be considered a consequence of the progression of erythroid differentiation [34,36,37].

We assayed whether follistatin and FLRG modulate the effects of activin A and BMP2 on human erythropoiesis. Follistatin or FLRG proteins canceled the activin-A-induced cell nuclear size decrease and inhibited the biological activity of activin A and BMP2, indicated by the normalization of the level of erythroid colonies. These regulators also block BMP2- and activin-A- induced expression of EPO-R and Hb. These results show that FLRG, like follistatin, constitutes a biological regulator of both activin A and BMP2 activity on human erythropoiesis. The modulation by follistatin and FLRG of activin A or BMP2 effects on GATA2 and EPO-R expression is different between these two inhibitory proteins. This indicates that despite their high structural homology [45], these regulators are not redundant. This is also consistent with their exclusive expression in some tissues, such as hematopoietic cells [29]. Moreover, the follistatin null mice display a severe phenotype [46], which speaks favorably for the absence of a complete functional redundancy between follistatin and FLRG.

Different type I and II receptors of the TGFβ family generate numerous receptor complexes for the same ligand, thereby creating combinatorial signaling and indicating that one molecule can couple with multiple type I receptors. The specificity of intracellular signaling, determined by type I receptors and their interaction with a specific Smad protein [39,40], may explain the different effects of the two follistatin family members. Indeed, we suggest that the addition of follistatin and FLRG might perturb different or identical ligand–receptor complexes in a different manner, thereby leading to distinct modulation of the biological effect mediated by the TGFβ family members. This is supported by the different modes of association of follistatin and FLRG with activin and the distinct mode of interaction of follistatin with BMP from that of follistatin with activins [27,30,47]. These physical interactions have been shown to inhibit activin and BMP signaling pathways.

In the presence of both activin and BMP2, we observed distinct effects on the modulation of the different parameters, as in the presence of the molecules alone. In particular, the effect on erythroid colonies appears to be the same as that with BMP2 alone. These results suggest that a competition might exist between activin and BMP2. Such a competition has been previously described for activin and inhibin effects on human erythroid differentiation [48]. This competition, leading to an antagonistic effect of inhibin on activin-mediated biological response, is the result of a competition between the two molecules for their binding to the same type II receptor. Interestingly, unlike activin A, which signals via combinations of activin receptors of type I and II, BMPs interact not only with BMP type I and II receptors but also with type IA and IIA activin receptors [49,50]. It is therefore possible that BMP2 competes with activin A when binding to the same receptor, and, as regards the difference in their respective affinity for the same receptor, a fine-tuning of signaling can therefore be

achieved. Thus, it now would be interesting to determine which receptor complexes are involved in the specific signaling of either activin or BMP2 during erythroid cell commitment.

Finally, the addition of BMP4 on primary cells has no significant effect except for a decrease in GATA2 and an increase in Hb and Bcl-XL expression. This indicates that BMP4 alone did not significantly affect human erythroid maturation, in terms of erythroid colony formation. Inversely to what has been described in *Xenopus* embryo [20] and rhesus monkey embryonic stem cells [12], BMP4 induces a decrease in GATA2 expression. This apparent discrepancy can reflect the various cell types assayed. Indeed, experiments were performed using embryonic pluripotent stem cells, while we used adult human primary $CD34^+$ cells. Unsurprisingly, this suggests that the program of gene expression involved in the formation of blood cells is different between embryonic and adult hematopoiesis, thus leading to apparently opposite effects on GATA2 expression. When added simultaneously with activin A, BMP4 decreased the effects of activin A on EPO-R and $p27^{Kip1}$ expression, accentuated its effects on cell nuclear size and Bcl-XL expression, and had no effect on GATA2 and Hb expression. At the functional level, both late BFU-E and CFU-E colonies are no longer amplified in the presence of BMP4 and activin A. These data might reflect a cooperation between BMP4 and activin A in accelerating human erythropoiesis as previously described in *Xenopus* embryonic stem cells [18]. This is expressed by an acceleration of erythroid maturation to a stage where cells, no longer able to form colonies, have downregulated the expression of EPO-R, decreased their nuclear size, and accumulated Hb and Bcl-XL, altogether suggesting a more differentiated erythroid maturation stage.

In summary, we showed a regulation of the erythroid differentiation in the presence of activin A and BMP2. The follistatin and FLRG molecules modulate, in a nonredundant way, the effects of activin A and BMP2 on various parameters of erythroid maturation. Finally, the simultaneous addition of activin A with BMP molecules indicates that BMP2 may compete and overcome, while BMP4 may cooperate and amplify, activin A effects. In this work, we pointed out potential target genes of the activin and BMP signaling pathways. This might therefore shed a new light on the molecular mechanisms of differences between activin A and BMP signaling and also contribute to the understanding of their role in the regulation of human erythropoiesis.

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References

[1] S.E. Jacobsen, J.R. Keller, F.W. Ruscetti, P. Kondaiah, A.B. Roberts, L.A. Falk, Bidirectional effects of transforming growth factor beta (TGF-beta) on colony-stimulating factor-induced human myelopoiesis in vitro: Differential effects of distinct TGF-beta isoforms, Blood 78 (1991) 2239–2247.

[2] H.W. Snoeck, S. Weekx, A. Moulijn, F. Lardon, M. Lenjou, G. Nys, P.C. Van Ranst, D.R. Van Bockstaele, Z.N. Berneman, Tumor necrosis factor alpha is a potent synergistic factor for the proliferation of primitive human hematopoietic progenitor cells and induces resistance to transforming growth factor beta but not to interferon gamma, J. Exp. Med. 183 (1996) 705–710.

[3] P.C. Van Ranst, H.W. Snoeck, F. Lardon, M. Lenjou, G. Nijs, S.F. Weekx, I. Rodrigus, Z.N. Berneman, D.R. Van Bockstaele, TGF-beta and MIP-1 alpha exert their main inhibitory activity on very primitive $CD34^+CD38^-$ cells but show opposite effects on more mature $CD34^+CD38^+$ human hematopoietic progenitors, Exp. Hematol. 24 (1996) 1509–1515.

[4] H.E. Broxmeyer, L. Lu, S. Cooper, R.H. Schwall, A.J. Mason, K. Nikolics, Selective and indirect modulation of human multipotential and erythroid hematopoietic progenitor cell proliferation by recombinant human activin and inhibin, Proc. Natl. Acad. Sci. USA 85 (1988) 9052–9056.

[5] N.L. Frigon Jr., L. Shao, A.L. Young, L. Maderazo, J. Yu, Regulation of globin gene expression in human K562 cells by recombinant activin A, Blood 79 (1992) 765–772.

[6] M. Shiozaki, R. Sakai, M. Tabuchi, T. Nakamura, K. Sugino, H. Sugino, Y. Eto, Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis, Proc. Natl. Acad. Sci. USA 89 (1992) 1553–1556.

[7] J. Yu, K.E. Dolter, Production of activin A and its roles in inflammation and hematopoiesis, Cytokines Cell Mol. Ther. 3 (1997) 169–177.

[8] M. Shiozaki, M. Kosaka, Y. Eto, Activin A: A commitment factor in erythroid differentiation, Biochem. Biophys. Res. Commun. 242 (1998) 631–635.

[9] B.M. Johansson, M.V. Wiles, Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development, Mol. Cell Biol. 15 (1995) 141–151.

[10] M. Bhatia, D. Bonnet, D. Wu, B. Murdoch, J. Wrana, L. Gallacher, J.E. Dick, Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells, J. Exp. Med. 189 (1999) 1139–1148.

[11] G. Bhardwaj, B. Murdoch, D. Wu, D.P. Baker, K.P. Williams, K. Chadwick, L.E. Ling, F.N. Karanu, M. Bhatia, Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation, Nat. Immunol. 2 (2001) 172–180.

[12] F. Li, S. Lu, L. Vida, J.A. Thomson, G.R. Honig, Bone morphogenetic protein 4 induces efficient hematopoietic differentiation of rhesus monkey embryonic stem cells in vitro, Blood 98 (2001) 335–342.

[13] C.J. Marshall, A.J. Thrasher, The embryonic origins of human haematopoiesis, Br. J. Haematol. 112 (2001) 838–850.

[14] L.I. Zon, Self-renewal versus differentiation, a job for the mighty morphogens, Nat. Immunol. 2 (2001) 142–143.
[15] L. Shao, N.L. Frigon Jr., A.L. Young, A.L. Yu, L.S. Mathews, J. Vaughan, W. Vale, J. Yu, Effect of activin A on globin gene expression in purified human erythroid progenitors, Blood 79 (1992) 773–781.
[16] H. Uchiyama, M. Asashima, Specific erythroid differentiation of mouse erythroleukemia cells by activins and its enhancement by retinoic acids, Biochem. Biophys. Res. Commun. 187 (1992) 347–352.
[17] Y. Miyanaga, R. Shiurba, M. Asashima, Blood cell induction in *Xenopus* animal cap explants: Effects of fibroblast growth factor, bone morphogenetic proteins, and activin, Dev. Genes Evol. 209 (1999) 69–76.
[18] T.L. Huber, Y. Zhou, P.E. Mead, L.I. Zon, Cooperative effects of growth factors involved in the induction of hematopoietic mesoderm, Blood 92 (1998) 4128–4137.
[19] M.V. Wiles, B.M. Johansson, Analysis of factors controlling primary germ layer formation and early hematopoiesis using embryonic stem cell in vitro differentiation, Leukemia 11 (3) (1997) 454–456.
[20] M. Maeno, P.E. Mead, C. Kelley, R.H. Xu, H.F. Kung, A. Suzuki, N. Ueno, L.I. Zon, The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in *Xenopus laevis*, Blood 88 (1996) 1965–197.
[21] V.C. Broudy, N. Lin, M. Brice, B. Nakamoto, T. Papayannopoulou, Erythropoietin receptor characteristics on primary human erythroid cells, Blood 77 (1991) 2583–2590.
[22] M. Leonard, M. Brice, J.D. Engel, T. Papayannopoulou, Dynamics of GATA transcription factor expression during erythroid differentiation, Blood 82 (1991) 1071–1079.
[23] P. Pedrazzoli, M.A. Bains, R. Watson, J. Fisher, T.G. Hoy, A. Jacobs, c-myc and c-myb oncoproteins during induced maturation of myeloid and erythroid human leukemic cell lines, Cancer Res. 49 (1989) 6911–6916.
[24] J.M. Freyssinier, C. Lecoq-Lafon, S. Amsellem, F. Picard, R. Ducrocq, P. Mayeux, C. Lacombe, S. Fichelson, Purification, amplification and characterization of a population of human erythroid progenitors, Br. J. Haematol. 106 (1991) 912–922.
[25] T. Nakamura, K. Takio, Y. Eto, H. Shibai, K. Titani, H. Sugino, Activin-binding protein from rat ovary is follistatin, Science 247 (1990) 836–838.
[26] T. Yamashita, S. Takahashi, E. Ogata, Expression of activin A/erythroid differentiation factor in murine bone marrow stromal cells, Blood 79 (1992) 304–307.
[27] S. Iemura, T.S. Yamamoto, C. Takagi, H. Uchiyama, T. Natsume, S. Shimasaki, S. Sugino, N. Ueno, Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo, Proc. Natl. Acad. Sci. USA 95 (1998) 9337–9342.
[28] L. Bartholin, V. Maguer-Satta, S. Hayette, S. Martel, M. Gadoux, L. Corbo, J.P. Magaud, R. Rimokh, Transcription activation of FLRG and follistatin by activin A, through Smad proteins, participates in a negative feedback loop to modulate activin A function, Oncogene 21 (2002) 2227–2235.
[29] V. Maguer-Satta, L. Bartholin, S. Jeanpierre, M. Gadoux, S. Bertrand, S. Martel, J. Magaud, R. Rimokh, Expression of FLRG, a novel Activin A ligand, is regulated by TGFβ and during hematopoiesis, Exp. Hematol. 29 (2001) 301–308.
[30] K. Tsuchida, K.Y. Arai, Y. Kuramoto, N. Yamakawa, Y. Hasegawa, H. Sugino, Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-beta family, J. Biol. Chem. 275 (2000) 40788–40796.
[31] L. Bartholin, V. Maguer-Satta, S. Hayette, S. Martel, M. Gadoux, S. Bertrand, L. Corbo, C. Lamadon, A.M. Morera, J.P. Magaud, R. Rimokh, FLRG, an activin-binding protein, is a new target of TGF-beta transcription activation through Smad proteins, Oncogene 20 (2001) 5409–5419.
[32] Y. Miyamoto, M. Kosaka, Y. Eto, H. Shibai, S. Saito, Effect of erythroid differentiation factor on erythroid differentiation and proliferation of K-562 cells, Biochem. Biophys. Res. Commun. 168 (1990) 1149–1156.
[33] S.T. Koury, M.J. Koury, M.C. Bondurant, Morphological changes in erythroblasts during erythropoietin-induced terminal differentiation in vitro, Exp. Hematol. 16 (1988) 758–763.
[34] E. Fibach, D. Manor, A. Oppenheim, E.A. Rachmilewitz, Proliferation and maturation of human erythroid progenitors in liquid culture, Blood 73 (1989) 100–103.
[35] M.O. Griffin, J.C. Nelson, N.G. Abraham, Early protooncogene expression during hemin-induced differentiation of human erythroleukemic cells, Leuk. Res. 14 (1990) 997–1006.
[36] D. Josefsen, J.H. Myklebust, J. Lonno, M. Sioud, H.K. Blomhoff, E.B. Smeland, Differential expression of bcl-2 homologs in human CD34(+) hematopoietic progenitor cells induced to differentiate into erythroid or granulocytic cells, Stem Cells 18 (2000) 261–272.
[37] F.F. Hsieh, L.A. Barnett, W.F. Green, K. Freedmen, I. Matushansky, A.I. Skoultchi, L.L. Kelley, Cell cycle exit during terminal erythroid differentiation is associated with accumulation of p27(Kip1) and inactivation of cdk2 kinase, Blood 96 (2000) 2746–2754.
[38] S.H. Pope, E. Fibach, J. Sun, K. Chin, G.P. Rodgers, Two-phase liquid culture system models normal human adult erythropoiesis at the molecular level, Eur. J. Haematol. 64 (2000) 292–303.
[39] P. ten Dijke, K. Miyazono, C.H. Heldin, Signaling inputs converge on nuclear effectors in TGF-beta signaling, Trends Biochem. Sci. 25 (2000) 64–70.
[40] E. Piek, C.H. Heldin, P. Ten Dijke, Specificity, diversity, and regulation in TGF-beta superfamily signaling, FASEB J. 13 (1999) 2105–2124.
[41] P.A. Gregoli, M.C. Bondurant, The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin, Blood 90 (1997) 630–640.
[42] Y. Zermati, B. Varet, O. Hermine, TGF-beta1 drives and accelerates erythroid differentiation in the epo-dependent UT-7 cell line even in the absence of erythropoietin, Exp Hematol. 28 (2000) 256–266.
[43] T. Sato, T. Maekawa, S. Watanabe, K. Tsuji, T. Nakahata, Erythroid progenitors differentiate and mature in response to endogenous erythropoietin, J. Clin. Invest. 106 (2000) 263–270.
[44] P. Ikonomi, C.E. Rivera, M. Riordan, G. Washington, A.N. Schechter, C.T. Noguchi, Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation, Exp. Hematol. 28 (2000) 1423–1431.
[45] S. Hayette, M. Gadoux, S. Martel, S. Bertrand, I. Tigaud, J.P. Magaud, R. Rimokh, FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders, Oncogene 16 (1998) 2949–2954.
[46] M.M. Matzuk, N. Lu, H. Vogel, K. Sellheyer, D.R. Roop, A. Bradley, Multiple defects and perinatal death in mice deficient in follistatin, Nature 374 (1995) 360–363.
[47] A.L. Schneyer, D.A. Rzucidlo, P.M. Sluss, W.F. Crowley Jr., Characterization of unique binding kinetics of follistatin and activin or inhibin in serum, Endocrinology 135 (1994) 667–674.
[48] J.J. Lebrun, W.W. Vale, Activin and inhibin have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human erythroid differentiation, Mol. Cell Biol. 17 (1997) 1682–1691.
[49] P. ten Dijke, H. Yamashita, T.K. Sampath, A.H. Reddi, M. Estevez, D.L. Riddle, H. Ichijo, C.H. Heldin, K. Miyazono, Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4, J. Biol. Chem. 269 (1994) 16985–16988.
[50] H. Yamashita, P. ten Dijke, D. Huylebroeck, T.K. Sampath, M. Andries, J.C. Smith, C.H. Heldin, K. Miyazono, Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects, J. Cell Biol. 130 (1995) 217–226.